Available online at www.sciencedirect.com

SciVerse ScienceDirect

Physics Procedia 33 (2012) 872 – 876

Physics Procedia

2012 International Conference on Medical Physics and Biomedical Engineering

Expression and Bioinformatics Analysis of Pectate Lyase Gene from Bacillus subtilis521

Jing Xiao[1,2], Fu-Ping Lu[1*], Yu Li[1], Jin-Ting Li[1]

College of Biotechnology,
[1]Tianjin University of Science & Technology,Tianjin, People's Republic of China
[2] College of Life Science and Engineering ,Qiqihar University
Qiqihar, People's Republic of China
lfp@tust.edu.cn

Abstract

In order to exploit new genetic resources, Pectate lyase(PEL) gene was amplified by PCR using the genome DNA from an alkaline Bacillus subtilis521. The PCR product was inserted into pET22b(+) vector. The recombinant plasmids were cloned in E.coli DH5α and then expressed in E.coli BL21. When cultured in the optimized medium, the positive clones E.coli BL21(pET22b(+)pel)showed intracellular pectate lyase activity of 90.0 U/mL. It was indicated that we had obtained the correct PEL gene. The pel has an open reading frame of 1263 nucleotides and codes for a product of 420 amino acids with a calculated molecular mass of 45.5 kD. Based on computer assisted analysis, a signal peptides and two conserved domains were revealed. The sequence analysis for PEL showed that it shares 26-82% homology with other strains in GenBank. In addition, the advanced structure of PEL were also predicted and analysed. This study will help to the experimental design of PEL fermentation and production purification and enzyme evolution.

Keywords- Bacillus subtilis; Pectate lyase gene; transform; bioinformatic; sequence analysis

Introduction

Pectin is a heteropolysaccharide composed of 1,4-α-linked galacturonate chains existed in a wide variety of higher plants. Alkaline pectate lyase, which can cleave the bonds between galacturonate unit, plays a powerful role in degradation of pectin more than hydrolase, especially in alkaline environment. Recently, pectate lyase has received more attention because of its ability to degrade highly esterified pectin with no prior action of other pectinases[1]. In order to adapt to the range of applications, particularly in the food, pharmaceutical and detergent industries, pectate lyase resources should be exploited fully and efficiently[2,3]. In this paper, more than 100 strains which can produce alkaline pectate lyase were

 doi:10.1016/j.phpro.2012.05.148

screened from Tianjin alkaline soil. Among these selected strains, *Bacillus megaterium* and *Bacillus subtilis* were the dominate classifications in comparison with *Serratia marcescens*, *Enterobacter*. In all the cases , enzyme activity of strain 521 reached the maximum 15.0U/mL when cultured in optimization medium .The strain 521 was then tested by the means of morphologic methods, physiological and biochemical reaction , molecular biology methods and was identified as *Bacillus subtilis* ,termed *Bacillus subtilis* 521. The optimal temperature and pH of the crude pectate lyase enzyme were about 60℃ and pH10.0. Enzyme activity could remain at least 50% when incubated 1h at 60℃ and pH below 11.0. Based on the excellent properties, superexpression is one of the chief purposes. The probability of the gene encoding pectate lyase used in the breeding of gene engineering bacteria could be evaluated by the construction of *E.coli* engineering bacteria. Otherwise, computer assisted prediction and analysis of sequence and structure of the gene would be valuable to the research and application of the target enzyme.

Materials and methods

A. Bacterial Strains, Plasmids and Culture Conditions

Bacillus subtilis 521 containing the pectate lyase gene (*pel*) was isolated earlier from an alkaline soil sample collected in China. *E.coli* DH5α and *E.coli* BL21 were used as hosts for the cloning and expression of *pel*. Plasmid pET22b(+) was chosen as the vector for transforming and cloning. Bacteria was grown in Luria-Bertani(LB) broth. If necessary, ampicillin(100μg/mL) was added to the growth medium.

B. Cloning of B. subtilis 521 Pectate Lyase (PEL) Gene

The gene encoding part of PEL was amplified by polymerase chain reaction(PCR) with the *B. subtilis* 521 genomic DNA as template and appropriate mix primers A and B. Genomic DNA was prepared as described by Saito and Miura [4]. Primer A, 5'-CCCAAGCTTATGAAAAA AGTGATGTTAGC-3' (HindⅢ restriction sit: AAGCTT), Primer B, 5'-CCGCTCGAGTTAATTTAATTTACCCGCA C-3 ' (Xhol restriction sit: CTCGAG) were designed based on the nucleotide sequence of *B.subtilis* subsp. subtilis str. 168 (NCBI, NC00964). PCR was performed using probest DNA polymerase (TaKaRa).

C. Construction of Recombination Plasmid

The amplified PCR fragment was digested with both HindⅢ and XhoI and the purified fragment was cloned into HindⅢ-XhoI restriction sits of the pET22b(+) vector, resulting in pET22b(+)*pel*, which was introduced into the cloning host *E.coli* DH5α competance cell by chemical process. The amplified recombinant plasmid was transformed into the expression host *E. coli* BL21 formed *E.coli* BL21(pET22b(+)*pel*). DNA fragments were recovered with the Agarose Gel DNA Purification Kit (TaKaRa) and ligated with Ligator kit (TaKaRa). The positive recombinant clone was analyzed by digestion of restriction endonuclease.

D. Expression and Collection of Enzyme (PEL)

Transformed cells harbouring PEL gene were grown overnight in 5mL LB supplemented with ampicillin (100μg/mL). Then 2mL of the overnight culture was inoculated into 100mL LB supplemented with ampicillin (100μg/mL) in a 500mL flask and incubated with shaking at 180 rpm for about 3h at 30℃ until the OD_{600} reached 1.0. Pectate lyase was then induced by adding 1mmol/L IPTG. Cells were harvest after 6h induction time by centrifugation (4000g,4℃,10min) and then suspended in 5mL lysis buffer (0.2mol/L glycin-NaOH, pH9.0,10mg/mL lysozyme) After incubated 1h at 37℃,cell debris was removed by centrifugation(10,000g,4℃,15min)

E. Enzyme Activity Measurements

Pectate lyase activity was measured by quantifying the production of C_4 and C_5 unsaturated polygalacturonic acid spectrophotometrically at 235nm[5].The reaction mixture containing 2mL 0.2% Poly-D-galacturonic acid,0.44mmol/L $CaCl_2$ in 0.2mol/L glycin-NaOH buffer (pH9.0) and 20μL of

enzyme solution, was incubated at 45℃ for 15min.The reaction was stopped with addition of 3mL of 0.03mol/L phosphoric acid. One unit of PEL was defined as the amount of enzyme which releases 1μmol of unsaturated polygalacturonic per minute at 45℃ and pH9.0.The calculation of the enzyme activity was based on the formula 1. X is the enzyme activity and N is dilution ratio of the sample (U/mL)

$$X=3.6232\cdot N\cdot OD_{235}. \quad (1)$$

F. Computer-assisted Design and Analysis

Primer Premier5.0 and BioEdit7.0.5.3 were used for DNA analysis. SignalP 3.0 server was used for the prediction of signal peptide cleavage sites. Multiple sequence alignments and protein homology search were performed using DNAMAN and BLAST. For protein structure analysis, ExPASy Proteomics Server and 3D-JIGSAW Protein Comparative Modeling Server were used.

Results and discussion

G. Gene Expression and Pectate Lyase Activity

The gene *pel* from *Bacillus subtilis* 521 were amplified by polymerase chain reaction. Gene *pel* with molecular weight of 1263bp were transformed into *E.coli* BL21 formed *E.coli* BL21（pET22（+）*pel*） and expressed under the induction of IPTG. When cultured in the optimized medium , *E.coli* BL21 （pET22（+）*pel*）showed intracellular activity of 90.0 U/mL and extracellular activity of 2.5U/mL. It was indicated that we had obtained the correct PEL gene. Sequenced and analyzed showed that a 1263 base pairs long of *pel*, including the start coden ATG and the terminer coden TAA, was obtained using PCR method and was cloned into pET22b(+) vector successfully.

H. Gene Sequence and Amino Acid Analysis

The gene *pel* encoded 420 amino acid sequence with pI8.26 and molecular weight of 4.5kD. SDS-PAGE analysis showed that the molecular weight of expressed products PEL were about 4.3kDa(not shown) . ExPASY analysis showed that the protein is composed of 20 kinds of amino acids with 37% negatively charged residues and 39% positively charged residues. Potential methylase sites included residues GATC 2-C (DAM modification , BclI et al restriction site) and CCWGG 2-C(DCM modification, Acc65I et al restriction site) were not found in our sequence. SignalP analysis indicated that the sequence has a signal peptide of 21 amino acids long. The recognition site for signal peptidase with a characteristic sequence (polar N and C terminus, hydrophobic H-area) ended with micromolecule amino acid. Results of SignalP-NN and SignalP-HMM both discovered the max cleavage site probability between pos.21-22.

I. Sequence Similarity

The sequence of *pel* from *B.subtilis* 521 are 98.89% identical to the sequence of *Bacillus subtilis* subsp. subtilis str. 168(NCBI, NC00964).The different bases 135:A-G,136:T-G,147:C-G,261:G-A,285:C-T,528:C-T,555:C-T, 594:G-A,669:T-C,756:C-T,778:C-T,861:T-A,981:T-C,1230: T-C and a different amino acid residue S46A（Ser-Ala）were confirmed. Aligmented with other PEL gene resources, the target gene showed 26-82% similarity. Compared the PEL from *B.subtilis* to those from *B. licheniformis* and *B. amyloliquefaciens*, the similarity is 50-80%.Further research found that there were two catalytic domains: 45-QTDASNGANYITMS-258,296-VQRAPRVRFGQVHVYN
-312 in PEL. As expected of pectate lyases, Ca^{2+} ions were probably required for the activity of the new PEL based on the prediction of binding-site for Ca^{2+} .Experiment proved *pel* could be used for construction and breeding high-yield gene engineering bacteria in appropriate expression system.

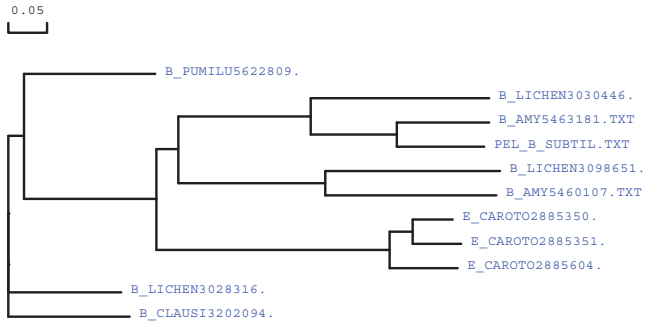

Figure 1. Homology tree of genes of pectate lyase from different strains

ExPASy Proteomics Server and 3D-JIGSAW Protein Comparative Modeling Server were used for prediction .3D structure of PEL from *B.subtilis* 521(Fig.2)had high homology to those from other bacteria.

Figure 2. Predicted 3D structure of pectate lyase from *Bacillus subtilis*521.

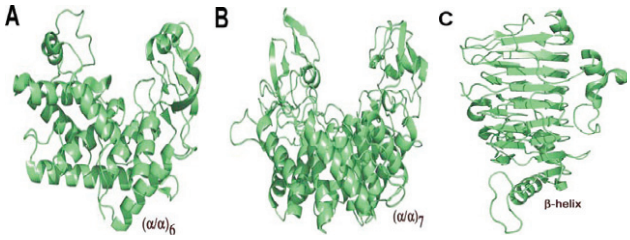

Figure 3. Structural conservation of the active sites within pectate lyases displaying diverse folds The unique folds for three different pectate lyase families are shown in panels A-C. A, (α/α)6 toroid (Protein Data Bank code 1GXO). B, (α/α)7 barrel (Protein Data Bank code 2V8I). C,β-helix (Protein Data Bank code 2EWE)

The polypeptide backbone folds into a large cylinder, termed a parallel β -helix. Different sizes loops extend out of the central area and probably confer function. Among these loops, the largest one folds into a small domain consisting of β -helix and α -helix. *B.subtilis* and gram negative such as bacteria *Erwinia* , fungi such as *Aspergillus* have the similar higher structure relating to a pectate lyase superfamily. In addition to the β-helix structure, rare forms (α/α)6 and (α/α)7 were found in *Cellvibrio japonicus* and *Yersinia enterocolitica* separately [6,7].

The pectate lyase gene *pel* from alkaline *B.subtlis*521 was cloned and expressed by construction of engineering strain. This pectate lyase possesses a 21-residue signal peptide that is cleaved during export, following which the protein is secreted into periplasm. Computer assisted analysis of sequence and structure would be benifit for further research and application [8,9]. For example, Ca^{2+} should be added during the course of pectate lyase fermentation in order to meet the ions demands. When purified enzyme was needed, characteristics such as molecular and pI will play a large role in separation and purification. The prediction of higher structure and function fragments will ensure better progress in the genetic engineering.

References

[1] Margarita Soriano, Ana Blanco, Pilar Dı!az and F. I. Javier Pastor. An unusual pectate lyase from a Bacillus sp. with high activity on pectin:cloning and characterization, Microbiology,2000, Vol.146,pp. 89-95

[2] Basu S,Saha M N,Chattopadhyay D,et al. Degumming and characterization of ramie fibre using pectate lyase from immobilized Bacillus pumilus DKS1. Letters in applied microbiology,2000, Vol.48, pp.593-597

[3] Jain S,Durand H,et al. Production of extracellular pectinase enzymes by a mutant .of Penicillium occitanis. Appl. Microbiol. Biotechnol, 1990, Vol.34,pp.308-312.

[4] Saito,H & Miura,K. Preparation of transforming deoxyribonucleic acid by phenol treatment.Biochim.Biophys.Acta 1963,vol.72,pp.619-629

[5] Collmer,A.,J.l.Ried, Moumt.M.S. Assay methods for pectic enzymes.Methods Enzymol.1988,vol.161,pp.329-399

[6] Sathyanarayana. N,Gummadi,T.Panda. Purification and biochemical properties of microbial pectinases-a reviw.Process Biochemistry. 2003,vol.38,pp.987-996

[7] Yuji Natada, Kazuhiro Saito,Kenzo Koike,Tadashi Yoshimatsu, Tadahiro Ozawa,Tohru Kobayashi and Susumu lto. Deduced amino-acid sequence and possible catalytic residued of a novel pectate lyase from an alkaliphilic strain of Bacillus.Eur.J.Biochem. 2000,vol.267, pp.2268-2275

[8] Pickersgill R,Jenkins J,Harris G,et al.The structure of Bacillus subtilis pectate lyase in complex with calcium.Nat.Struct.Biol.,1994,pp.717-723

[9] HUANG Zhipeng,GUAN Chunhong,HUANG Biwang,QIU Junzhi &GUAN Xiong.Cloning and Bioinformatics of a New Gene, cry1Ab17 from Bacillus thuringiensis. Chin J Appl Environ Biol, 2006, Vol.12,pp.64-67